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J. Clinton Bailey II
University of Southern Mississippi

Sabine Heinhorst
University of Southern Mississippi, sabine.heinhorst@usm.edu

Gordon C. Cannon
University of Southern Mississippi, Gordon.Cannon@usm.edu

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Accuracy of Deoxynucleotide Incorporation by Soybean Chloroplast DNA Polymerases Is Independent of the Presence of a 3′ to 5′ Exonuclease[1]

J. Clinton Bailey II, Sabine Heinhorst, and Gordon C. Cannon*

Department of Chemistry and Biochemistry, University of Southern Mississippi, Hattiesburg, Mississippi 39406

DNA polymerase was purified from soybean (*Glycine max*) chloroplasts that were actively replicating DNA. The main form (form I) of the enzyme was associated with a low level of 3′ to 5′ exonuclease activity throughout purification, although the ratio of exonuclease to polymerase activity decreased with each successive purification step. A second form (form II) of DNA polymerase, which elutes from DEAE-cellulose at a higher salt concentration than form I, was devoid of any exonuclease activity. To assess the potential function of the 3′ to 5′ exonuclease in proofreading, the fidelity of deoxynucleotide incorporation was measured for form I DNA polymerase throughout purification. Despite the steadily decreasing ratio of 3′ to 5′ exonuclease to polymerase activity, the extent of misincorporation by form I enzyme remained unchanged during the final purification steps, suggesting that the exonuclease did not contribute to the accuracy of DNA synthesis by this polymerase. Fidelity of form I DNA polymerase, when compared with that of form II, revealed a higher level of misincorporation for form I enzyme, a finding that is consistent with the exonuclease playing little or no role in exonucleolytic proofreading.

Chloroplasts contain multiple copies of a circular, covalently closed DNA molecule of about 150 kb, termed the plastome. This molecule encodes many of the gene products required for the organelle to carry out photosynthesis and for organellar housekeeping. The levels of ctDNA appear to be highly regulated in a developmental and tissue-specific manner, with high plastome copy numbers being observed in photosynthetically active leaves and very low copy number in roots (reviewed by Heinhorst and Cannon, 1993). Little is known about the mechanism or control of ctDNA replication, but all gene products required to replicate the plastome are encoded by nuclear genes that are expressed in the cytoplasm and imported into the organelle. To date, no genes have been identified that encode ctDNA replication proteins, but several putative ctDNA replication enzymes such as topoisomerases, helicase, primase, and DNA polymerases have been characterized biochemically (reviewed by Heinhorst and Cannon, 1993).

Sequence and arrangement of genes located on the plastome are highly conserved throughout the plant kingdom (Palmer, 1985). Rates of evolutionary sequence changes in ctDNA have been estimated to be 2 times lower than those of nuclear DNA (Wolfe et al., 1987), which has led to the speculation that this remarkable DNA sequence stability might be based on efficient postreplicative DNA repair and/or a highly accurate DNA replication mechanism operative in the chloroplast. Although our current knowledge of plant organellar DNA repair is scant, several ctDNA polymerase activities have been isolated and characterized from *Chlamydomonas reinhardtii* (Wang et al., 1991), spinach (Sala et al., 1980; Keim and Mosbaugh, 1991), pea (McKown and Tewari, 1984), and soybean (Heinhorst et al., 1990). In general, the enzymes from higher plants are similar in molecular size, stimulation by salt, resistance to aphidicolin, and sensitivity to N-ethylmaleimide and can be classified as eukaryotic γ-type DNA polymerases, with the possible exception of the enzyme from pea (McKown and Tewari, 1984). ctDNA polymerases from most plants are reported to consist of a single catalytic subunit with a mol wt between 85,000 and 110,000 (Sala et al., 1980; McKown and Tewari, 1984; Heinhorst et al., 1990; Keim and Mosbaugh, 1991), although activity gel analysis of the enzyme from *C. reinhardtii* indicated the presence of more than one catalytic subunit (Wang et al., 1991). Whether these differences can be attributed to proteolytic degradation of the enzyme during purification, partial subunit dissociation, or the variable nature of activity gels when applied to organelle DNA polymerases is unknown.

Association of nucleases with ctDNA polymerase activity has been observed during purification of the enzymes from pea (McKown and Tewari, 1984) and *C. reinhardtii* (Wang et al., 1991); however, this association apparently was fortuitous, since the final purified fraction of the DNA polymerase was devoid of any nuclease activity in the case of the green alga and of pea (McKown and Tewari, 1984; Wang et al., 1991). Keim and Mosbaugh (1991) characterized a 3′ to 5′ exonuclease that co-purified with the ctDNA polymerase from spinach through various chromatography steps. The nuclease has a slightly higher affinity for mismatched 3′ ends than for fully base-paired substrates

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* Corresponding author; e-mail gcannon@whale.st.usm.edu; fax 1-601-266-5829.

Abbreviations: dNTP, deoxyribonucleotide triphosphate; ssDNA, single-stranded DNA; TMP, thymidine monophosphate; TTP, thymidine triphosphate.

and seems to reside on a 20,000-kD polypeptide that is apparently only weakly associated with the DNA polymerase catalytic subunit. The fact that the polymerase was isolated from tissue that was replicating little or no ctDNA calls into question the in vivo function of the DNA polymerase observed, and the low levels of the enzyme present made an assessment of the exonuclease's role in the DNA synthesis process difficult. The tight association of a 3′ to 5′ exonuclease activity with animal (Wernette and Kaguni, 1986; Kunkel and Soni, 1988; Wernette et al., 1988; Insdorf and Bogenhagen, 1989; Kaguni and Olson, 1989; Kunkel and Mosbaugh, 1989; Longley and Mosbaugh, 1991; Olson and Kaguni, 1992) and plant (Meissner et al., 1993) mtDNA polymerases, on the other hand, is well documented.

The 3′ to 5′ exonucleases that are complexed with or are part of DNA polymerases often contribute to the overall accuracy of DNA synthesis by providing a proofreading function that corrects base substitution errors made by the DNA polymerase (Wang, 1991). It was, therefore, of interest to determine whether the fidelity of ctDNA polymerase from higher plants is modulated by an associated 3′ to 5′ exonuclease activity. We have reproducibly observed in chloroplasts that were purified from photoautotrophic soybean suspension culture cells two forms of DNA polymerase that are distinguishable by their different elutions from DEAE-cellulose and by the inability of form II to bind to phosphocellulose. In this paper we report that a low, but persistently present, 3′ to 5′ exonuclease activity co-purifies with form I ctDNA polymerase, whereas form II is devoid of an exonuclease activity. We have taken advantage of the fact that only one of the otherwise biochemically indistinguishable DNA polymerase forms contains the nuclease activity to assess the role of the exonuclease in polymerization fidelity. Using a gel electrophoresis misincorporation assay, we have compared the two enzyme forms and have followed the fidelity of form I throughout purification.

MATERIALS AND METHODS

Chemicals

Deoxynucleotides and (+)M13 mp18 DNA were from Pharmacia LKB (Piscataway, NJ). Oligonucleotides were purchased from several commercial sources. DEAE-cellulose (DE 52) and phosphocellulose (P-11) were purchased from Whatman through Fisher Scientific (Pittsburgh, PA). ssDNA cellulose, PMSF, and *p*-toluene sulfonyl fluoride came from Sigma. DTT was purchased from Fisher Scientific. Radioactive nucleotides, [γ-^{32}P]ATP at 3000 Ci/mmol and [^{3}H]TTP at 83 Ci/mmol, were purchased from NEN Dupont (Wilmington, DE).

Isolation of Chloroplasts and Purification of ctDNA Polymerase Forms I and II

The green SB-1 cell line of *Glycine max* var Corsoy (Horn et al., 1983) was maintained as previously reported (Heinhorst et al., 1990). Chloroplasts were isolated and the organellar DNA polymerase was purified essentially as described by Heinhorst et al. (1990) with the following modifications: Pooled form I DNA polymerase fractions from the phosphocellulose chromatography step were dialyzed against three changes of buffer C (50 mM Tris-HCl [pH 8], 10% [v/v] glycerol, 100 mM ammonium sulfate, 0.1 mM EDTA, 0.1 mM *p*-toluene sulfonyl fluoride, and 0.1 mM PMSF) and loaded onto a 5-mL heparin-agarose column cartridge (Bio-Rad) equilibrated in buffer C. The column was washed with 5 column volumes of buffer C and the DNA polymerase activity was eluted with a linear 100 to 500 mM ammonium sulfate gradient in buffer C. Active fractions were pooled for further purification by chromatography on ssDNA cellulose as previously described (Heinhorst et al., 1990). Form II ctDNA polymerase was passed over a heparin-agarose cartridge following chromatography on DEAE-cellulose.

Glycerol Gradient Centrifugation

Fractions eluting from ssDNA cellulose and containing active form I ctDNA polymerase were pooled and sedimented through a 15 to 35% glycerol gradient as detailed previously (Heinhorst et al., 1990), except that 250 mM $(NH_4)_2SO_4$ was used instead of KCl.

DNA Polymerase Assay

DNA polymerase activity was determined in a 50-μL assay containing 50 mM Tris-HCl (pH 8), 125 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 140 μg/mL BSA, 125 μg/mL activated DNA, and 30 μM each of dATP, dGTP, dCTP, and TTP/[^{3}H]TTP with the specific radioactivity ranging from 370 to 37,000 cpm/pmol deoxynucleotides. Deoxynucleotide incorporation was quantitated by precipitation of nucleic acids onto glass fiber filters with TCA, followed by liquid scintillation counting as previously described (Heinhorst et al., 1990). A unit of DNA polymerase activity is defined as the amount of enzyme required to incorporate 1 pmol of deoxynucleotides into acid-insoluble material in 30 min at 37°C.

3′ to 5′ Exonuclease Assays

Precipitation Assay with [^{3}H]TMP-Labeled Activated DNAs

Activated DNA was 3′ end labeled with [^{3}H]TMP to a specific radioactivity of 5000 to 6000 cpm/μg (Olson and Kaguni, 1992). A unit of 3′ to 5′ exonuclease activity is defined as the amount of enzyme necessary to release 1 pmol of free deoxynucleoside monophosphate in 30 min at 37°C, as determined by acid precipitation on glass fiber filters.

Gel Assay with 5′ End-Labeled Oligonucleotide

Gel-purified oligonucleotide T41 (5′-GAC TCT AGA GGA TCC CCG-3′) was labeled at the 5′ end using T4 polynucleotide kinase by standard published methods (Maniatis et al., 1989). Exonuclease assays, containing in a 20-μL reaction volume 50 mM Tris-HCl (pH 8), 7.5 mM $MgCl_2$, 1 mM DTT, and 0.7 pmol of 5′ end-labeled oligonucleotide, were incubated for 2 h at 37°C. To stop the

reaction, 10 μL of formamide containing 0.1% bromphenol blue, 0.1% xylene cyanol, and 10 mM EDTA were added and the samples were heated to 95°C prior to loading a 10-μL aliquot onto a 20% polyacrylamide gel (0.5 mm × 16 cm × 20 cm) in 7 M urea and buffer (90 mM Tris-borate, 2.5 mM EDTA [pH 8.3]). Electrophoresis in this buffer proceeded for 3 to 6 h at 12 mA. The gel was dried and an autoradiograph produced using Kodak X-OMAT AR film and a Dupont Cronex intensifying screen. To quantitate band intensities, autoradiographs were digitized with a Cohu High Performance CCD camera and the image was analyzed using Image 1.47 (National Institutes of Health, Bethesda, MD). Manipulation of the digitized image was limited to background subtraction by the two-dimensional rolling ball method. The percentage excision was calculated by setting the area of the band representing untreated T41 as 100% (blank value) and subtracting the area of the residual T41 in the enzyme assay samples from the blank value before dividing the resulting difference by the blank value.

Gel Misincorporation Assay

The gel misincorporation assay is a modification of a previously published assay that was designed to detect single-base misincorporations into a singly primed, ssDNA template (Hillebrand et al., 1984). Briefly, 1 to 10 μg of single-stranded circular (+)M13 mp18 DNA and 100 pmol of the complementary oligonucleotide T41 (5′ end labeled with ^{32}P) were mixed in buffer E (10 mM Tris-HCl [pH 8], 100 mM NaCl), heated to 100°C for 10 min, and cooled to room temperature for several hours. The primed template (+)M13 mp18:[^{32}P]-T41 was separated from unannealed, labeled primer by size exclusion chromatography on Sephacryl S-200 (column size 18 × 1 cm) in buffer E. Fractions of 250 μL were collected and the radiolabel in the void volume was detected by liquid scintillation counting. Those fractions that contained primed template were pooled and concentrated to a minimum volume using a Microcon 30 concentrator (Amicon, Beverly, MA).

Misincorporations were detected by allowing the ctDNA polymerase to elongate the primer of the (+)M13 mp18:[^{32}P]-T41 template in the presence of three of the four dNTPs (minus reaction). A 25-μL reaction contained 50 mM Tris-HCl (pH 8), 7.5 mM $MgCl_2$, 1 mM DTT, 50 mM NaCl, and 30 μM each of three dNTPs. Separate reactions, each lacking a different one of the four dNTPs, were analyzed for each enzyme sample. The reactions are referred to by the name of the absent nucleotide (e.g. the assay lacking dATP is the −A reaction). Assays with a complete set of dNTPs and without any dNTPs served as positive and negative controls, respectively. The reactions were terminated by addition of 15 μL of formamide containing 0.1% bromphenol blue, 0.1% xylene cyanol, and 10 mM EDTA. Electrophoresis and autoradiography were as described for the exonuclease assay.

The DNA polymerase can faithfully incorporate dNTPs into the nascent strand until it encounters the first position on the template that requires incorporation of the missing deoxynucleotide (first stop site), at which point it will pause. A labeled oligonucleotide intermediate that has been elongated to a size equal to primer plus the number of correctly incorporated nucleotides accumulates and can be visualized by autoradiography of a denaturing polyacrylamide gel. Any elongation of the oligonucleotide primer beyond the first stop site can be attributed to misincorporation of an incorrect dNTP.

Analysis of band intensities was as described for the exonuclease gel assay. The relative levels of misincorporation were calculated by dividing the sum of the peak areas representing bands larger than the first stop site band by the sum of the peak areas corresponding to all bands larger than and equal to the first stop site band. The highest level of misincorporation of each minus reaction for the form I polymerase was set to one and the average misincorporation frequencies calculated by averaging the data obtained for the four minus reactions.

RESULTS

Purification of 3′ to 5′ Exonuclease with Forms I and II ctDNA Polymerase

DNA polymerase and 3′ to 5′ exonuclease activities were readily observed in the cleared chloroplast extract, as were a variety of nonspecific nuclease activities that complicated quantitation of 3′ to 5′ exonuclease activity in the crude lysate. Most of the exonuclease activity was not retained on DEAE-cellulose and either passed through or was washed off the column with low salt loading buffer (not shown). As previously observed (Heinhorst et al., 1990), two forms of DNA polymerase were retained on DEAE-cellulose with form I eluting at 140 mM and form II at 250 mM NaCl. Multiple peaks of 3′ to 5′ exonuclease activity, as measured by the precipitation assay, eluted from the column across the entire salt gradient (Fig. 1A).

Pooled, active fractions that contained form I DNA polymerase, as expected, eluted from a phosphocellulose column with a linear 20 to 400 mM potassium phosphate gradient as a single peak (Fig. 1B). Although some fraction of the total 3′ to 5′ exonuclease activity co-purifies with the DNA polymerase peak at 180 mM potassium phosphate, the majority of the exonuclease activity elutes as a single sharp peak at 130 mM potassium phosphate (Fig. 1B).

Further purification of form I DNA polymerase on heparin-agarose yielded two peaks of 3′ to 5′ exonuclease activity upon developing the column with a linear 100 to 500 mM ammonium sulfate gradient. The first elutes at 160 mM ammonium sulfate, whereas the second co-purifies with the DNA polymerase activity at 200 mM salt (Fig. 1C). The association of the 3′ to 5′ exonuclease activity with the highly purified form I DNA polymerase was further assessed by chromatography on ssDNA cellulose, followed by centrifugation in a 15 to 35% glycerol gradient. At this point, it became necessary to use the gel assay (see "Materials and Methods"), which offers greater sensitivity than the precipitation assay, to detect the low levels of remaining exonuclease activity. Following step elution of the ctDNA polymerase from ssDNA cellulose with 500 mM sodium chloride, a single peak of 3′ to 5′ exonuclease

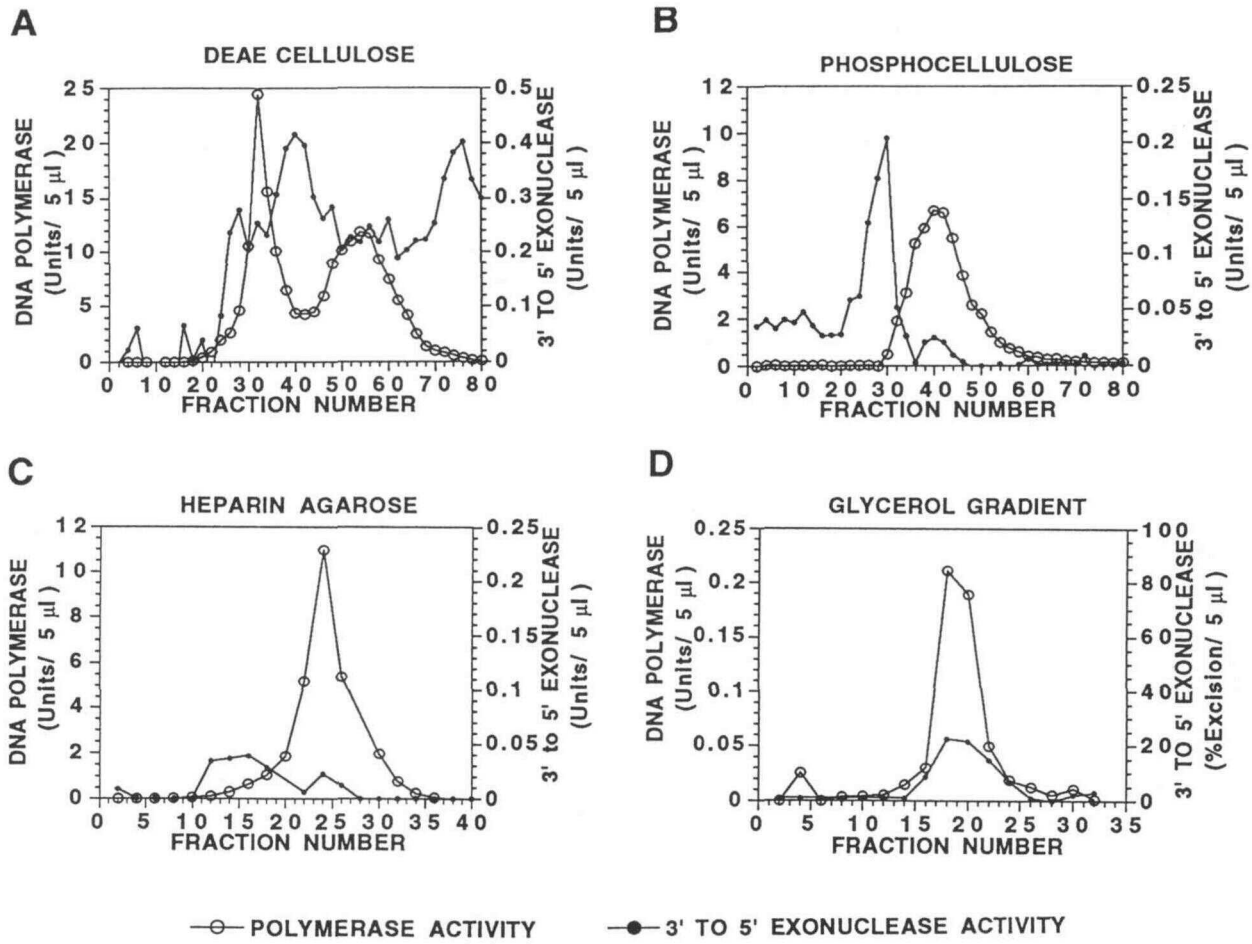

Figure 1. Co-purification of form I ctDNA polymerase and 3′ to 5′ exonuclease activity. Shown are elution profiles from DEAE-cellulose (A), phosphocellulose (B), and heparin-agarose (C). D, Glycerol gradient profile. The polymerase activity was measured by incorporation of [^{3}H]TMP into activated DNA. The 3′ to 5′ exonuclease activity was quantitated by the precipitation assay (A–C) and by the gel assay (D).

activity co-purified with the enzyme (not shown). The exonuclease activity also remained associated with form I ctDNA polymerase through glycerol gradient centrifugation (Fig. 1D), and no other 3′ to 5′ exonuclease activity was detected in the gradient.

Form II ctDNA polymerase purified on DEAE-cellulose, when subjected to chromatography on heparin-agarose, was found to be devoid of any detectable 3′ to 5′ exonuclease activity, using both precipitation and gel assays (not shown). To directly compare the relative levels of 3′ to 5′ exonuclease activity associated with both highly purified forms of ctDNA polymerase, equal amounts of form I and II activity were allowed to react with ^{32}P-labeled oligonucleotide T41 for 2 h in the absence of added nucleotides. Reaction products were resolved by electrophoresis through a 20% polyacrylamide gel. The autoradiograph shown in Figure 2 confirms that form I DNA polymerase activity co-purifies with a 3′ to 5′ exonuclease activity, as detected by the decrease in size of the oligonucleotide substrate brought about by this enzyme fraction (lane A). Form II ctDNA polymerase is not associated with a nucle-

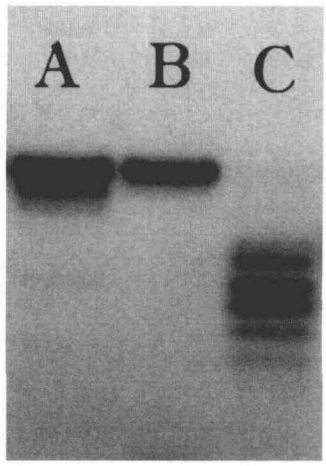

Figure 2. Comparison of 3′ to 5′ exonuclease activity of ctDNA polymerase forms I and II. Exonuclease assays were performed with equal amounts (units) of polymerizing activity. A, Form I; B, form II of ctDNA polymerase; C, *E. coli* polymerase I.

ase activity, as evidenced by the integrity of the 5′ end-labeled substrate even after 2 h of incubation (lane B).

Characterization of the 3′ to 5′ Exonuclease Activity That Is Associated with Form I ctDNA Polymerase

The 3′ to 5′ exonuclease associated with form I ctDNA polymerase from soybean requires the presence of millimolar concentrations (1–7.5 mM) of Mg for optimal activity. Mn can substitute for Mg (optimum concentration 0.25 mM). The divalent cation optima determined for the exonuclease are in the range of those determined previously for maximal polymerase activity (Heinhorst et al., 1990).

The effect of ammonium sulfate and sodium chloride on DNA polymerase and 3′ to 5′ exonuclease activity shown in Figure 3A confirms stimulation of the polymerizing activity by increasing salt concentrations that was previously observed for γ-type DNA polymerases from animal and plant organelles (Heinhorst et al., 1990; Wang et al., 1991; Meissner et al., 1993). The 3′ to 5′ exonuclease activity associated with form I ctDNA polymerase, on the other hand, is strongly inhibited by salt (Fig. 3B) and Figure 3, C and D, indicates that the observed effect is due to ionic strength. An ionic strength of 0.35 is sufficient to totally inhibit the 3′ to 5′ exonuclease while stimulating the polymerase activity almost 6-fold.

Ratio of Form I Polymerase to 3′ to 5′ Exonuclease Activity throughout Purification

Table I gives the ratio of polymerase to 3′ to 5′ exonuclease activity throughout the purification of form I enzyme from soybean chloroplasts. The ratio of polymerase to exonuclease activity is not constant and increases with each successive purification step of the plant organellar enzyme, suggesting that the 3′ to 5′ exonuclease activity is contained on a dissociable subunit that is gradually removed from form I ctDNA polymerase during purification. Alternatively, most of the observed nuclease activity could be unrelated to the polymerase and be co-purifying fortuitously. Commercially available DNA polymerase I from *Escherichia coli* (Promega) displayed an approximately 8-fold higher exonuclease to polymerase activity ratio than form I of the enzyme from soybean chloroplasts (Table I).

Fidelity of Form I ctDNA Polymerase throughout Purification

The accuracy of nucleotide incorporation was assessed with the misincorporation assay described in "Materials

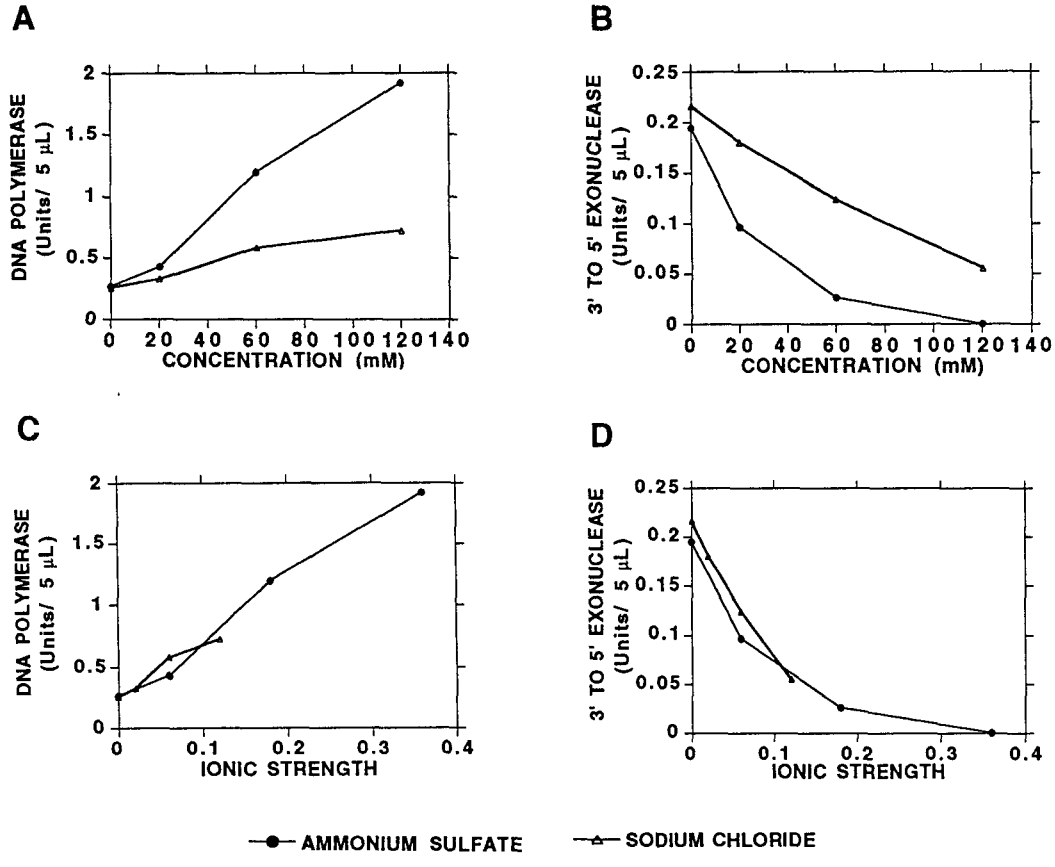

Figure 3. Effect of increasing salt concentration on form I ctDNA polymerase and 3′ to 5′ exonuclease activity. A, DNA polymerase activity; B, 3′ to 5′ exonuclease activity in the presence of increasing concentrations of ammonium sulfate and sodium chloride. The data from A and B were replotted to directly compare the effect of ionic strength on ctDNA polymerase activity (C) and 3′ to 5′ exonuclease activity (D).

Table I. *Ratio of 3′ to 5′ exonuclease to polymerase activity of form I ctDNA polymerase*

DNA polymerase and 3′ to 5′ exonuclease assays were performed as described in "Materials and Methods." Polymerase I from *E. coli* (Promega) is a highly purified enzyme fraction from an overproducing strain.

Fraction	3′ to 5′ Exonuclease Activity	DNA Polymerase Activity	Ratio of Polymerase to Exonuclease Activity
	units	*units*	
DEAE-cellulose	0.007	0.29	41:1
Phosphocellulose	0.012	4.1	341:1
Heparin-agarose	0.007	6.2	885:1
E. coli polymerase I	0.010	1.2	120:1

and Methods" for form I ctDNA polymerase at various stages of purification. Average misincorporation levels for the four deoxynucleotides were slightly lower in the chloroplast-cleared lysate than in the most highly purified heparin agarose fraction of form I ctDNA polymerase using equal amounts of DNA polymerase activity (units) (Table II). Because of the presence of nonspecific nucleases and the possible interference by form II polymerase in the chloroplast lysate, this value probably does not accurately reflect the fidelity of form I ctDNA polymerase in this crude fraction, particularly since form II consistently displayed higher polymerization fidelity than form I (Fig. 4). The polymerization fidelity of form I ctDNA polymerase increased somewhat with each purification step, although it is unclear whether this is a statistically significant trend or merely reflects no change in error frequencies of the enzyme. It is clear, however, that despite a more than 8-fold overall decrease in exonuclease to polymerase ratio during purification (Table II), form I ctDNA polymerase does not exhibit a concomitant increase in the average level of nucleotide misincorporation, as would be expected if the 3′ to 5′ exonuclease activity that is associated with this form of the enzyme contributed to the overall accuracy of DNA synthesis. The level of accuracy that was determined for form I ctDNA polymerase is within the range of, and quite possibly slightly higher than, that calculated for *E. coli* DNA polymerase I (Table II), an enzyme that features a prominent 3′ to 5′ exonuclease known to be actively involved in exonucleolytic proofreading during polymerization (Kornberg and Baker, 1992).

Table II. *Independence of polymerization fidelity of form I ctDNA polymerase on 3′ to 5′ exonuclease activity*

The relative misincorporation levels for all four deoxynucleotides were averaged as explained in "Materials and Methods." Polymerase I from *E. coli* (Promega) is a highly purified enzyme enzyme fraction from an overproducing strain.

Fraction	Ratio of Polymerase to Exonuclease Activity	Relative Misincorporation Level
Cleared lysate	ND[a]	0.55
DEAE	41:1	0.86
Phosphocellulose	341:1	0.74
Heparin-agarose	885:1	0.71
E. coli polymerase I	120:1	0.91

[a] ND, Not determined.

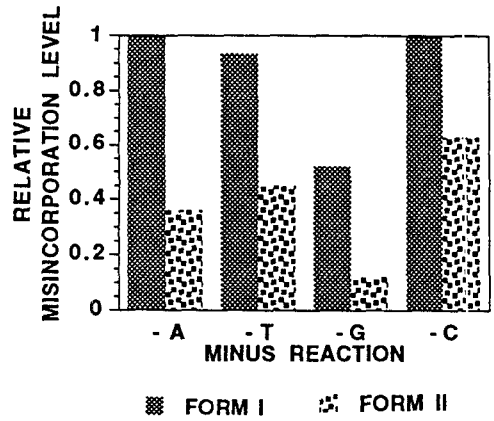

Figure 4. Relative levels of misincorporation by forms I and II of ctDNA polymerase. Equal amounts (units) of heparin-agarose-purified enzymes were compared using the gel assay described in "Materials and Methods."

Fidelity of Both Forms of ctDNA Polymerase

Figure 4 depicts the relative levels of nucleotide misincorporation determined for the two forms of ctDNA polymerase that were purified through the heparin agarose steps. Form I, which is associated with the 3′ to 5′ exonuclease activity, consistently exhibited a higher level of nucleotide misincorporation in each minus reaction (between 1.5- and 4.4-fold, depending on the missing deoxynucleotide) than form II, which is devoid of any nuclease activity. Although the data suggest preference for misincorporation of certain bases by the two forms of chloroplast polymerase, it must be emphasized that the experiment was designed to demonstrate trends and not to provide quantitative data. These data strongly suggest that in vitro the 3′ to 5′ exonuclease that co-purifies with form I polymerase does not seem to participate in proofreading.

DISCUSSION

A 3′ to 5′ exonuclease activity co-purifies with form I ctDNA polymerase obtained from a suspension cell line of soybean through five purification steps (Fig. 1), whereas form II of the enzyme, which is otherwise biochemically indistinguishable from form I (Heinhorst et al., 1990), is devoid of any nuclease activity (Fig. 2). The exonuclease activity associated with the ctDNA polymerase does not appear to be tightly bound, since a significant fraction can be separated from the DNA polymerase activity at each successive chromatography step (Fig. 1; Table I), with the removal of the majority of the 3′ to 5′ exonuclease from form I ctDNA polymerase occurring during chromatography on phosphocellulose. Keim and Mosbaugh (1991) previously described a 3′ to 5′ exonuclease that is associated with the DNA polymerase from spinach chloroplasts and apparently resides on a separate, smaller polypeptide that

is dissociable from the polymerase subunit by repeated sedimentation through glycerol gradients. The 3′ to 5′ exonuclease that is associated with form I DNA polymerase from soybean chloroplasts, on the other hand, does not separate from the catalytic polymerase subunit in glycerol gradients (Fig. 1). This could reflect a more extensive removal of easily dissociable or unrelated exonuclease activity from the soybean ctDNA polymerase than was achieved for the spinach enzyme prior to glycerol gradient centrifugation, or it could be indicative of a tighter association between both enzymes from the soybean organelle. Furthermore, it is unclear how many forms of ctDNA polymerase were present in the final enzyme fraction from spinach chloroplasts. Step elution of the DNA polymerase activity from DEAE-cellulose with 250 mM NaCl, as performed by Keim and Mosbaugh, and sedimentation through a glycerol gradient would not separate forms I and II of the DNA polymerase from soybean chloroplasts (Heinhorst et al., 1990).

Form II ctDNA polymerase readily separates from any detectable 3′ to 5′ exonuclease activity during heparin-agarose chromatography, although an exonuclease activity does co-purify with the polymerase during chromatography on DEAE-cellulose (Fig. 1). A 3′ to 5′ exonuclease activity was also not seen in the most highly purified fraction of the DNA polymerase from pea chloroplasts (McKown and Tewari, 1984) or with a γ-type DNA polymerase from *C. reinhardtii*, which might be located in the chloroplast of the alga (Wang et el., 1991). Nucleases that were present in the organellar enzyme preparations at earlier stages of the purification proved to be unrelated to the ctDNA polymerase.

The 3′ to 5′ exonuclease activity that is associated with form I ctDNA polymerase from soybean and with the enzyme from spinach chloroplasts is completely inhibited by salt concentrations of similar ionic strength (0.35), whereas the ctDNA polymerase activity from both plants is stimulated by increasing concentration, up to a maximum at 125 mM, of monovalent salt (Heinhorst et al., 1990; Keim and Mosbaugh, 1991). These results point to a sharp contrast between the DNA polymerases from higher plant chloroplasts and the analogous enzymes from animal mitochondria. The mitochondrial DNA polymerases from *Drosophila* embryos (Kaguni and Olson, 1989; Olson and Kaguni, 1992) and from porcine liver (Longley and Mosbaugh, 1991) co-purify with a 3′ to 5′ exonuclease activity. Both enzyme activities display practically identical monovalent salt optima and dependence on divalent cations. Furthermore, the polymerase to exonuclease ratio remained practically constant throughout the final purification steps of the enzymes from *Drosophila* (Kaguni and Olson, 1989) and *Xenopus laevis* (Insdorf and Bogenhagen, 1989), indicating a tight association between both enzymatic activities.

Since the 3′ to 5′ exonuclease associated with animal mtDNA polymerases has been shown to increase the overall accuracy of DNA synthesis by providing a proofreading function (Wernette and Kaguni, 1986; Kunkel and Soni, 1988; Insdorf and Bogenhagen, 1989; Kaguni and Olson, 1989), it was of interest to determine whether the analogous enzyme from chloroplasts also functions to remove misincorporated bases from the 3′ end of the newly synthesized DNA strand. Although the ratio of 3′ to 5′ exonuclease to polymerase activity of form I enzyme decreases steadily throughout purification (Table I), probably due to the dissociation of the exonuclease subunit, a concomitant increase in the level of nucleotide misincorporation was not observed (Table II). In fact, the fidelity of form I ctDNA polymerase remains constant through several purification steps, suggesting that the exonuclease does not contribute to faithful DNA synthesis. It should be mentioned that the reaction conditions, especially the salt concentration, that were chosen for the in vitro misincorporation assay were a compromise between the requirements of polymerase and exonuclease, while maintaining sufficient ionic strength to preserve the duplex structure of the DNA substrate. Surprisingly, form I ctDNA polymerase, which is associated with the 3′ to 5′ exonuclease activity, consistently misincorporates nucleotides at a higher level than does form II, which lacks the exonuclease activity, which lends further credit to the assumption that the two forms of ctDNA polymerase do not rely on a proofreading 3′ to 5′ exonuclease to contribute to their fidelity of polymerization. At this point, the basis for the higher fidelity of form II ctDNA polymerase is unclear, and the question of the relationship between the two forms of ctDNA polymerase observed in the soybean cell culture remains to be answered. Although it is assumed that at least one form of the enzyme is involved in plastome replication by virtue of the fact that no evidence for the presence of other DNA polymerase activities in chloroplasts has yet been obtained, it remains to be seen whether the two forms have different functions, such as replication versus repair, in the metabolism of the chloroplast genome. The elucidation of the mechanism(s) governing the faithful maintenance of the plastome, through accurate replication and/or an as-yet unidentified repair process, will undoubtedly require a better understanding of the polymerase(s) involved in these processes.

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